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# Sid4p-Cdc11p Assembles the Septation Initiation Network and Its Regulators at the *S. pombe* SPB

**Jennifer L. Morrell,[1] Gregory C. Tomlin,[1] Srividya Rajagopalan,[2] Srinivas Venkatram,[1] Anna S. Feoktistova,[1] Joseph J. Tasto,[1] Sapna Mehta,[1] Jennifer L. Jennings,[3] Andrew Link,[3] Mohan K. Balasubramanian,[2] and Kathleen L. Gould[1,*]**

[1]Howard Hughes Medical Institute and Department of Cell and Developmental Biology Vanderbilt University School of Medicine Nashville, Tennessee 37232

[2]Temasek Life Sciences Laboratory 1 Research Link The National University of Singapore Singapore 117604 Republic of Singapore

[3]Department of Microbiology and Immunology Vanderbilt University School of Medicine Nashville, Tennessee 37232

## Summary

**The *Schizosaccharomyces pombe* septation initiation network (SIN) triggers actomyosin ring constriction, septation, and cell division. It is organized at the spindle pole body (SPB) by the scaffold proteins Sid4p and Cdc11p. Here, we dissect the contributions of Sid4p and Cdc11p in anchoring SIN components and SIN regulators to the SPB. We find that Sid4p interacts with the SIN activator, Plo1p, in addition to Cdc11p and Dma1p. While the C terminus of Cdc11p is involved in binding Sid4p, its N-terminal half is involved in a wide variety of direct protein-protein interactions, including those with Spg1p, Sid2p, Cdc16p, and Cdk1p-Cdc13p. Given that the localizations of the remaining SIN components depend on Spg1p or Cdc16p, these data allow us to build a comprehensive model of SIN component organization at the SPB. FRAP experiments indicate that Sid4p and Cdc11p are stable SPB components, whereas signaling components of the SIN are dynamically associated with these structures. Our results suggest that the Sid4p-Cdc11p complex organizes a signaling hub on the SPB and that this hub coordinates cell and nuclear division.**

## Results

### Spg1p binds Directly to Cdc11p

The SIN is controlled by the activity of Spg1p, a GTPase that localizes to SPBs throughout the cell cycle [1]. We observed previously that Spg1p was not localized to SPBs in *cdc11* mutants [2]. This observation prompted us to test whether Cdc11p contained a binding site for Spg1p. In a two-hybrid assay, Spg1p interacted with full-length Cdc11p as well as the Cdc11p N terminus (amino acids 1–551) (Figure 1A). Furthermore, the N terminus of Cdc11p(1–660) produced in a coupled transcription/translation in vitro system that lacks yeast SPB components was able to bind bacterially produced MBP-Spg1p but not MBP alone (Figure 1B), indicating that Cdc11p and Spg1p interact directly. This interaction is likely to be independent of the nature of the nucleotide bound to Spg1p (Figure S1).

### Cdc16p Binds Cdc11p

Spg1p is maintained in an inactive state by the action of a two-component GAP comprising Cdc16p and Byr4p [3]. Despite its ability to bind Spg1p directly in vitro [3], Byr4p localization to the SPB is strictly dependent upon Cdc16p [4, 5]. Therefore, we examined whether Cdc16p interacted with Cdc11p or Sid4p to tether the GAP near its target G protein. By two-hybrid analysis, Cdc16p interacted with the N-terminal 660 amino acids of Cdc11p (Figure 1C) but not the Cdc11p C-terminal half or any fragment of Sid4p (data not shown). A variety of Cdc11p N-terminal fragments were produced in *E. coli* as MBP fusion proteins and tested for their ability to bind Cdc16p produced in a coupled transcription/translation reaction. Residues 488–660 of Cdc11p bound Cdc16p directly (Figure 1D). Although reproducible, this binding was not robust given that $<5\%$ of Cdc16p in the reaction bound Cdc11p. In cells, this interaction is likely to be stabilized by Spg1p and Byr4p.

### Cdc11p Interacts Directly with Sid2p

The Sid2-Mob1 protein kinase complex is another SIN component present at SPBs throughout the cell cycle [6–8]. After Cdk1p inactivation, Sid2p-Mob1p moves to the medial ring [6] to initiate cell division. Because Sid2p-Mob1p SPB localization depends upon Sid4p and Cdc11p but not Spg1p [6–8], we examined by two-hybrid analysis whether Sid2p and/or Mob1p interacted directly with Sid4p, Cdc11p, or both. No interactions were detected between Sid4p and either Sid2p or Mob1p or between Cdc11p and Mob1p (data not shown). However, an interaction between Sid2p and Cdc11p(1–660) was observed (Figure 2A). Cdc11p(488–660) was the smallest tested Cdc11p region that showed a positive interaction with Sid2p (Figure 2A). Using a similar strategy, we found that the N terminus but not the kinase domain of Sid2p directed its interaction with Cdc11p (Figure 2A). Indeed, a MBP fusion of Cdc11p(488–660) produced in bacterial cells but not MBP alone was able to bind in vitro-transcribed/translated Sid2p(1–207), indicating that Cdc11p and Sid2p interact directly (Figure 2B). We also tested whether Cdc11p and Sid2p could coimmunoprecipitate from *S. pombe* protein lysates. Sid2p-$Myc_{13}$ was detected in anti-HA immunoprecipitates from a *cdc11-*$HA_3$ *sid2-*$myc_{13}$ strain but not from *cdc11-* $HA_3$ or *sid2-* $myc_{13}$ single-tag strains (Figure 2C). Further confirmation of the ability of these proteins to interact in vivo came from analysis of Sid2p-TAP-associated proteins. After tandem affinity purification from the *sid2-TAP* strain, associated proteins were identified by

*Correspondence: kathy.gould@vanderbilt.edu

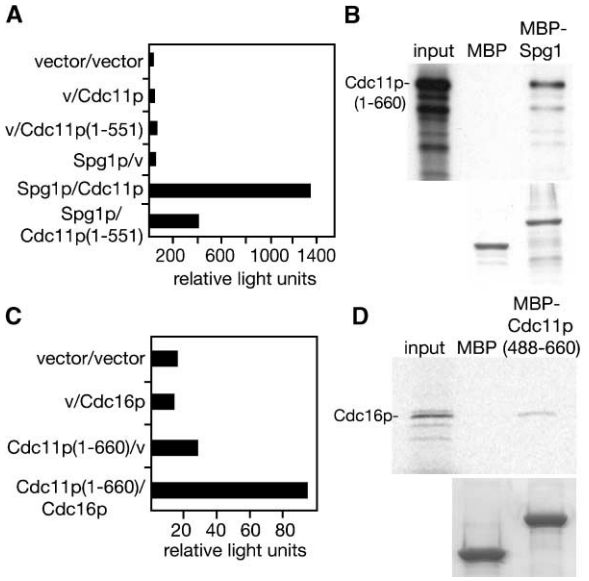


Figure 1. Cdc11p Binds Spg1p and Cdc16p

(A and C) Spg1p (A) or Cdc16p (C) bait plasmid was cotransformed with Cdc11p, Cdc11p(1–551), or Cdc11p(1–660) prey plasmids, and Leu+Trp+ transformants were scored for β-galactosidase activity in relative light units. V, vector control. (B) Approximately equal amounts of MBP or MBP-Spg1p were mixed with in vitro-translated Cdc11p(1–660). After washing, the proteins were detected by autoradiography (upper panel) or Coomassie staining (lower panel). A sample (4%) of in vitro-translated Cdc11p(1–660) before the binding experiment is shown in the input lane. (D) Approximately equal amounts of MBP or MBP-Cdc11p(488–660) were mixed with in vitro-translated Cdc16p. After being washed, the proteins were detected by autoradiography (upper panel) or Coomassie staining (lower panel). A sample (4%) of in vitro-translated Cdc16p before the binding experiment is shown in the input lane.

tryptic digestion, mass spectrometry, and database comparisons. In addition to Sid2p being identified at 38% sequence coverage, both Cdc11p and Mob1p were identified (at 15% and 18% sequence coverage, respectively), but Sid4p, Spg1p and other SIN components were not (data not shown). These results suggest that the interaction of Cdc11p with Sid2p-Mob1p is particularly stable.

Because Sid2p(1–207) interacts with Cdc11p, we tested whether this fragment of Sid2p was sufficient for SPB localization. GFP-Sid2p(1–207) localized to SPBs but not the medial ring (Figure 2D), indicating that the region of Sid2p that interacts with Cdc11p also directs its SPB localization. The Sid2p N terminus that binds Cdc11p also contains the binding site for Mob1p (data not shown and M.C. Hou and D. McCollum, personal communication). Using two-hybrid analysis, we could not define a region of Sid2p that interacted with Cdc11p but did not interact with Mob1p (data not shown). It is therefore possible that the binding regions for Cdc11p and Mob1p overlap or that these short Sid2p constructs did not fold properly.

Because the Cdc11p C terminus binds Sid4p but lacks the Sid2p binding site, we predicted that its overproduction would abolish Sid2p SPB localization by competing with endogenous Cdc11p for the Sid4p binding site at the SPB. Consistent with this prediction, Sid2p-GFP was readily detectable at SPBs and the medial ring in cells expressing low levels of Cdc11p(631–1045) but was absent from SPBs in cells overproducing this fragment (Figure 2E). Furthermore, GFP-Mob1p was also absent from poles in cells overproducing Cdc11p(631–1045) (Figure 2E), suggesting that Mob1p requires the N terminus of Cdc11p and Sid2p for its SPB localization.

### Sid4p and Cdc11p Are Stable SPB Components

We next investigated the relative turnover rates of Sid4p, Cdc11p, Sid2p, and Spg1p at SPBs by FRAP (Figure 3). GFP-tagged functional variants of these proteins were photobleached at the SPBs in both interphase and mitotic cells, and the rates of fluorescent recovery were determined. In both interphase and mitotic cells, Spg1p and Sid2p recovered fluorescence very rapidly after photobleaching (≤1 min) (Figures 3C–3E). In contrast, the recovery of Sid4p and Cdc11p was significantly slower. In mitotic cells, Cdc11p and Sid4p signals were faintly detected only at 6.5 ± 1.91 min and 10.4 ± 2.97 min, respectively (Figures 3A, 3B, and 3E). Cdc11p and Sid4p exchange was somewhat faster in interphase cells (3.3 ± 1.1 and 4.4 ± 0.9 min, respectively) (Figure 3E), and this likely reflects the processes of SPB growth and duplication. Even 20 min after photobleaching, <30% of fluorescence was recovered in the case of Sid4p and Cdc11p, as opposed to the near-complete recovery in the case of Sid2p and Spg1p (Figures 3A–3D and data not shown). Similar results were observed when Z-series of Sid4p-GFP- and Cdc11p-GFP-expressing cells were obtained (Figure S3 in the Supplemental Data available with this article online), confirming that the failure to detect fluorescence recovery of Sid4-GFP and Cdc11p-GFP in these experiments did not result from SPBs moving to different focal planes. These experiments further indicate that Sid4p and Cdc11p function as a scaffold through which other SIN components, such as Sid2p and Spg1p, associate dynamically.

### Sid4p-Cdc11p Interact with Protein Kinases that Regulate SIN Activity

In addition to organizing SIN components at the SPB, we reasoned that Sid4p-Cdc11p might recruit SIN regulators in addition to Dma1p, a checkpoint protein that associates with Sid4p and inhibits SIN signaling [10]. Plo1p, the *S. pombe* polo-like kinase, is required for actin ring formation and septation [11, 12], and SIN mutants that depend upon elevated Plo1p activity for survival have been isolated [13]. Furthermore, when Plo1p is overproduced, Cdc7p is recruited transiently to the SPBs, and multiple rounds of septation are initiated [14]. To determine whether Plo1p interacted with Sid4p or Cdc11p at the SPB, we performed directed two-hybrid analyses. Although no interaction was detected between Plo1p and Cdc11p, the C terminus of Plo1p, including its polo-boxes, interacted with the N-terminal region of Sid4p (Figures 4A and 4B). Because Plo1p localizes to the SPB only during mitosis [12, 14], we reasoned that a complex containing these two proteins might be cell cycle stage-specific in vivo. Coimmunoprecipitation analyses were performed from *plo1-HA*$_3$

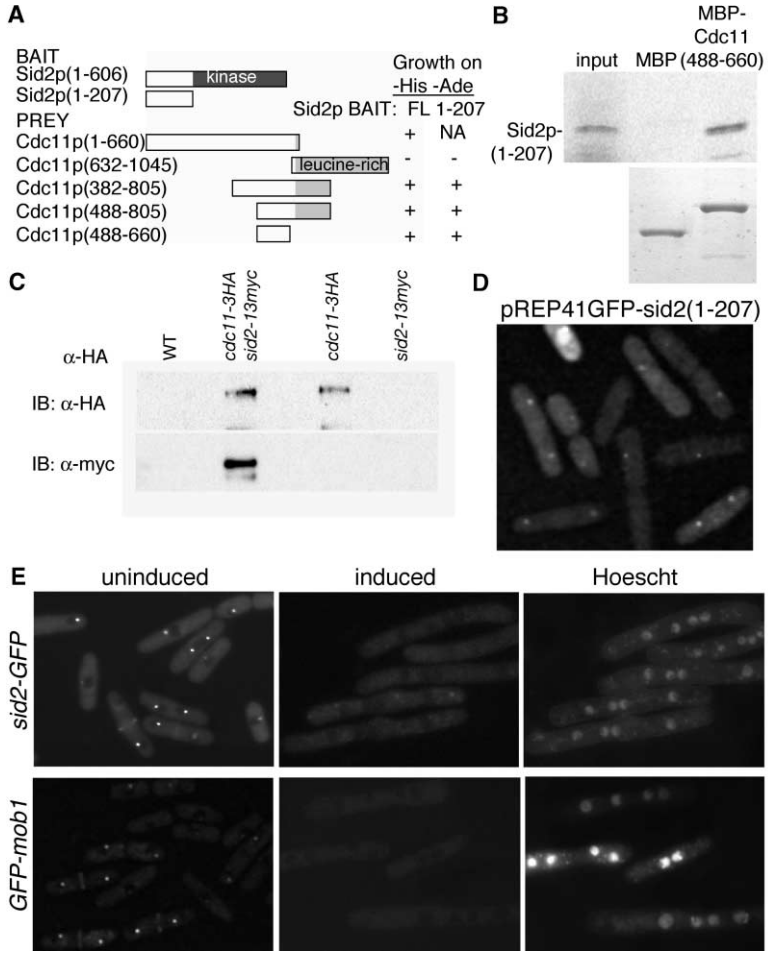


Figure 2. Cdc11p Binds Sid2p

(A) Sid2p(1–606) (full length) or Sid2p(1–207) bait plasmids were cotransformed with various Cdc11p prey plasmids, and Leu+Trp+ transformants were scored for positive interaction by growth on medium lacking His and Ade.

(B) Approximately equal amounts of MBP or MBP-Cdc11p(488–660) were mixed with in vitro-translated Sid2p(1–207). After being washed, the proteins were detected by autoradiography (upper panel) or Coomassie staining (lower panel). A sample (5%) of in vitro-translated Sid2p(1–207) before the binding experiment is shown in the input lane.

(C) Lysates from wild-type (KGY246), *cdc11-HA$_3$* (KGY3202), *sid2-myc$_{13}$* (KGY3736), and the double-tagged strain (KGY3924) were immunoprecipitated with anti-HA(12CA5) antibodies. After SDS-PAGE, samples were immunoblotted with either anti-HA or anti-Myc antibodies.

(D) Wild-type cells (KGY246) expressing GFP-Sid2p(1–207) were grown in the absence of thiamine. Images of live cells were captured.

(E) Overproduction of Cdc11p(631-1045) disrupts Sid2p and Mob1p SPB localization. Sid2p-GFP (KGY2945) and GFP-Mob1p (KGY3759) strains were transformed with pREP41cdc11(631–1045) and grown in the presence (uninduced, left panels) or absence (induced, middle and right panels) of thiamine. Images of live cells stained with Hoescht were captured.

*sid4-myc$_{13}$* cells that had been arrested in G2 with the *cdc25-22* mutation and then released to isolate cells in different cell cycle stages. Anti-HA immunoprecipitates contained Sid4p-Myc$_{13}$ when they were prepared from cells entering and exiting mitosis but not when they were prepared from G2 cells or cells that had completed division (Figure 4C). Sid4p-Myc$_{13}$ was not detected in immunoprecipitates lacking one of the epitope-tagged proteins. Furthermore, bacterially produced GST-Sid4(1–191) but not GST alone pulled out Plo1p-Myc$_{13}$ from cell lysates specifically (Figure 4D). These results establish that Plo1p exists in a physical complex with SIN components and suggest that Sid4p acts as a SIN-specific docking protein for Plo1p. As expected, Plo1p does not depend upon Sid4p for its SPB localization (data not shown). Because Plo1p is required for multiple SIN-independent aspects of mitosis, including mitotic spindle assembly [11, 15], Plo1p is expected to have additional interaction partners at the SPB. Indeed, another SPB protein with which Plo1p associates is Cut12p [16].

Whereas Plo1p positively influences SIN function, Cdk1p negatively regulates the SIN by preventing Sid1p-Cdc14p from binding the SPB and Sid2p-Mob1p relocalization [17–19]. Because Cdk1p also localizes to SPBs [20, 21], we tested whether Cdk1p or Cdc13p bound Sid4p or Cdc11p. By two-hybrid analyses, Cdc13p interacted with full-length Cdc11p and also the Cdc11p N terminus (Figure 4E). Also, Cdc11- Myc$_{13}$ was detected in anti-Cdc13p, anti-Cdk1p, and anti-Cdc11p immune but not preimmune sera immunoprecipitates, indicating that Cdc11p and Cdc13p associate in vivo (Figure 4F). The interaction of Cdk1p with Cdc11p was compromised in a *cdc13-117* mutant, which impairs Cdk1p-Cdc13p association, but not in *cdc13-A381V*, which does not ([22, 23]; Figure 4F). To determine whether the Cdc11p-Cdc13p interaction is direct, we produced a MBP fusion of Cdc11p(1–660) in bacterial cells and tested for its ability to bind in vitro-transcribed/translated Cdc13p. Cdc13p bound MBP-Cdc11p(1–660) but not MBP alone, indicating a direct association (Figure 4G).

The N terminus of Cdc11p(1–660) but not the C terminus of Cdc11p (569–1045) is phosphorylated on multiple serines by purified Cdk1p-Cdc13p in vitro (Figures S2A–S2C). All eight Cdk1p consensus phosphorylation sites lie in the Cdc11p N terminus, and when they are mutated to alanine residues, Cdk1p phosphorylation of Cdc11p (1–660) is abrogated (Figure S2D). Cdc11p-S8A complements both temperature-sensitive (Figure S2) and null (data not shown) alleles of *cdc11*, indicating that Cdk1p-Cdc13p phosphorylation is not essential for Cdc11p function. This finding is consistent with the Cdk1p independence of Cdc11p phosphorylation in vivo [24]. In any case, by analogy with the situation in *S. cerevisiae*, in

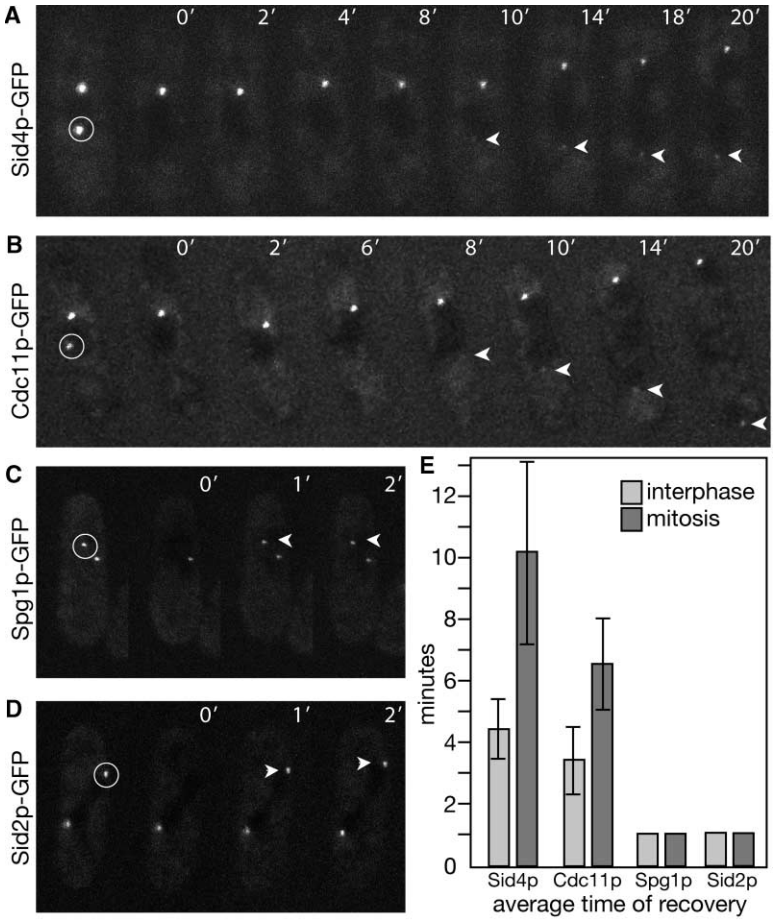


Figure 3. SIN Component Dynamics

(A) *sid4-GFP* (KGY2628), (B) *cdc11-GFP* (KGY3392), (C) *spg1-GFP* (KGY3197), and (D) *sid2-GFP* (KGY2945) cells were grown to mid-log phase in yeast extract (YE) medium and then photobleached in the zone marked by a white circle, corresponding to a spindle pole body. Recovery of fluorescence intensity was measured over time at 1 or 2 min intervals in a single focal plane of approximately 1.5 μm in depth. Images from a representative time course in a mitotic cell are shown for each strain. The time immediately after photobleaching is designated as 0 min. White arrowheads point to detectable fluorescence recovery from the photobleached SPB. (E) A graph showing the average time of fluorescence recovery for each strain. The total number of cells imaged was as follows: for *sid2-GFP*, 7 interphase and 8 mitotic; for *spg1-GFP*, 5 interphase and 10 mitotic; for *sid4-GFP*, 6 interphase and 7 mitotic; and for *cdc11-GFP*, 6 interphase and 8 mitotic.

which phosphorylation by Cdk1p restrains Cdc15p activity [25, 26], binding to Cdc11p would properly position Cdk1p-Cdc13p near Cdc7p (the Cdc15p homolog) and/or other targets to prevent downstream steps in SIN signaling until chromosome segregation has occurred. As with Plo1p, there are other binding partners for Cdk1p-Cdc13p at the SPB because in the absence of Cdc11p function, Cdc13p still localizes to the SPB (data not shown). Given that many mitotic events dependent upon Cdk1p activity continue to occur in SIN mutants, this is not an unexpected result.

## Conclusions

Sid4p and Cdc11p form a stable scaffold that organizes the SIN regulators, Plo1p, Cdk1p-Cdc13p, and Dma1p, in close proximity to all SIN components at the SPB (Figure 4H). Assembling such a signaling center is likely to be important for the tight coordination of cytokinesis with chromosome segregation, and it seems likely that other eukaryotes have similarly concentrated molecules involved in these processes at spindle poles to promote high signaling fidelity and tight coordination of mitotic events. Clearly, the *S. cerevisiae* counterpart of Cdc11p, Nud1p, binds at least a subset of the analogous proteins we have described here [26], and these are in close proximity to Spc72p (a possible Sid4p analog) that interacts with the Polo kinase and components of the γ-tubulin complex [27]. A remaining challenge is to identify the target(s) of the five protein kinases assembled at this signaling center and to establish how their activities affect the various steps of cytokinesis.

### Supplemental Data

Supplemental data, including three supplemental figures and Experimental Procedures, are available with this article online at http://www.current-biology.com/cgi/content/full/14/7/579/DC1/.

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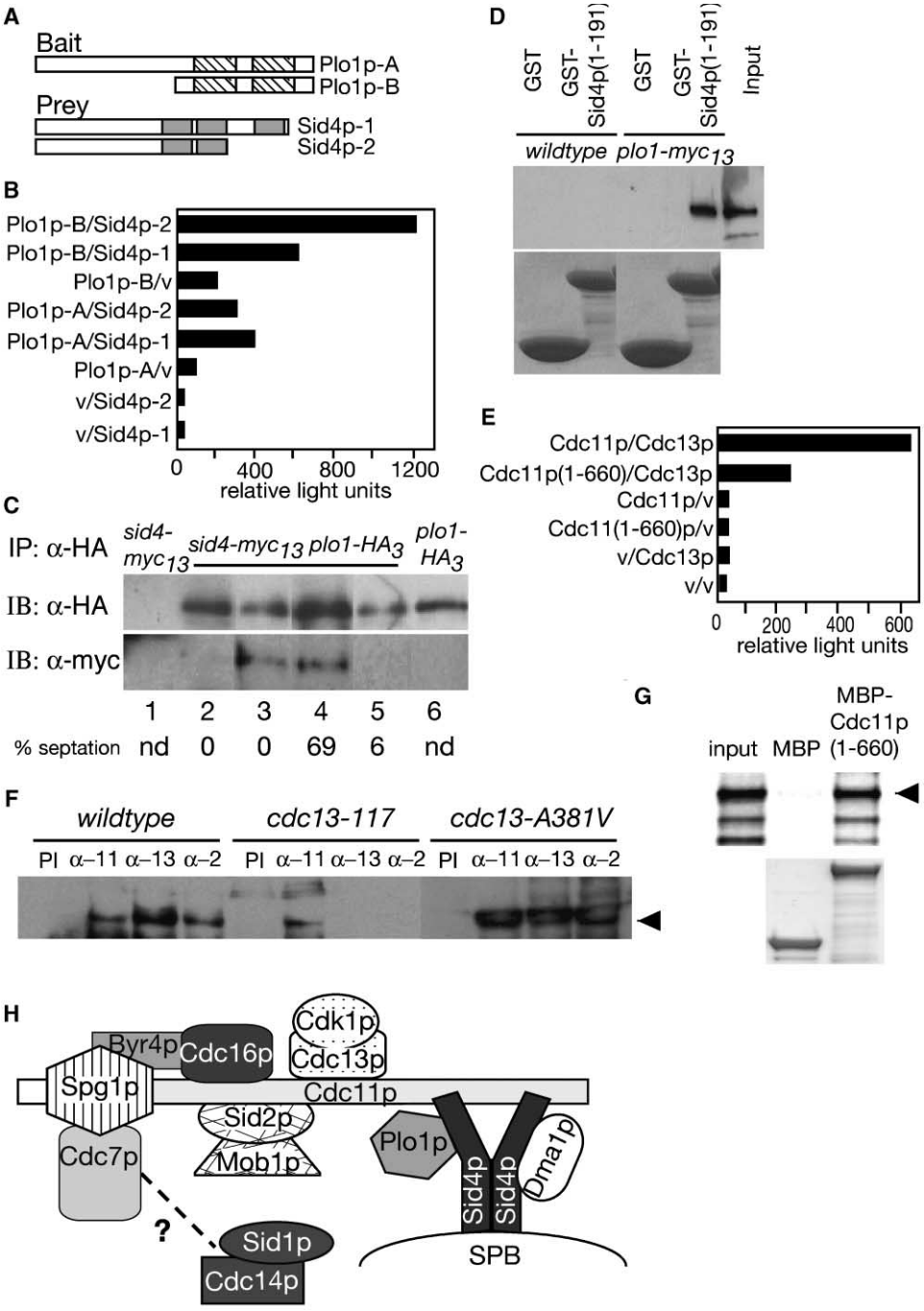


Figure 4. The Sid4p-Cdc11p Scaffold Recruits SIN Regulators

(A) Schematic representations of Plo1p and Sid4p constructs. The hatched boxes in Plo1p indicate the polo boxes, and the gray boxes in Sid4p denote the coiled-coil regions.

(B) The indicated bait and prey plasmids were cotransformed into PJ69-4A, LEU+ TRP+ transformants were assayed for β-galactosidase activity, and the results are depicted in relative light units. V, vector control.

(C) *plo1-HA$_3$ sid4-myc$_{13}$ cdc25-22* cells (KGY2211) were arrested at 36°C for 4 hr and released to 25°C, allowing synchronous entry into mitosis. Cells were obtained from the culture prior to release (lane 2) and at 30 (lane 3), 75 (lane 4), and 105 (lane 5) min after release. Cell synchrony was determined from the percentage of septating cells at each time point. These samples and those from the single-tagged strains *sid4-myc$_{13}$* (KGY2208) (lane 1) and *plo1-HA$_3$* (KGY1580) (lane 6) were lysed under native conditions. The lysates were subjected to anti-HA immunoprecipitations. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with either anti-HA (12CA5) (top panel) or anti-Myc (9E10) (bottom panel) antibodies. nd, not determined.

(D) GST and GST-Sid4p(1–191) were produced in bacteria, purified on glutathione beads, and incubated with protein lysates prepared from either *wild-type* (KGY246) or *plo1p-myc$_{13}$* (KGY779) cells. The bound complex was washed, and the presence of Plo1p-Myc$_{13}$ was detected by immunoblotting (top panel) and that of the GST fusion proteins by Coomassie staining (bottom panel).

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(E) The indicated *cdc11* bait plasmids (see Figure 2A for a schematic), *cdc13* prey plasmids, and control plasmids (v) were cotransformed into PJ69-4A, LEU+ TRP+ transformants were assayed for β-galactosidase activity, and the results are depicted in relative light units. V, vector control.

(F) *cdc11-myc$_{13}$* (*wild-type*), *cdc11-myc$_{13}$ cdc13-117*, and *cdc11-myc$_{13}$ cdc13-A381V* cells were grown at 25°C, shifted to 36°C for 4 hr, and lysed under native conditions. The lysates were subjected to immunoprecipitation with preimmune (PI) or immune anti-Cdc11p (α-11), anti-Cdc13p (α-13), or anti-Cdk1p (α-2) polyclonal antibodies. The immune complexes were resolved by SDS-PAGE and immunoblotted with anti-Myc (9E10) antibodies. The arrowhead indicates Cdc11p-Myc$_{13}$. The anti-Cdc11p antibodies only weakly immunoprecipitate Cdc11p (data not shown), accounting for the low recovery of Cdc11p-Myc with this antibody.

(G) Approximately equal amounts of MBP or MBP-Cdc11p(1-660) were mixed with in vitro translated Cdc13p. After washing, the proteins were detected by autoradiography (upper panel) or Coomassie staining (lower panel). The arrowhead corresponds to Cdc13p. A sample (approximately 5%) of in vitro-translated $^{35}$S-Cdc13p before the binding experiment is shown in the input lane.

(H) Model of SIN organization at the SPB. Although it seems probable that Sid1p-Cdc14p binds Cdc7p or another SIN component upon Cdk1p inactivation [17, 19], the positioning of Sid1p-Cdc14p within the signaling hub remains to be determined precisely.